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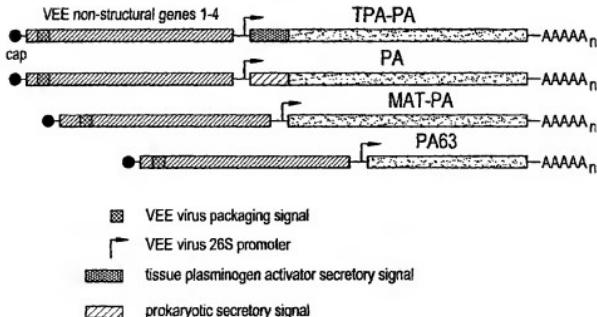
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(54) Title: ANTHRAX VACCINE

(57) Abstract: Using the nontoxic protective antigen PA protein from *B. anthracis*, a method and composition for use in inducing an immune response which is protective against anthrax in subjects is described.

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TITLE OF THE INVENTION

Anthrax Vaccine

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FIELD OF THE INVENTION

This invention relates to vaccines for bacterial toxins from *Bacillus anthracis*.

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INTRODUCTION

Anthrax is a disease cause by the sporulating bacteria *Bacillus anthracis*. Humans working with animal products are at risk from contracting anthrax.

- 20 Areas such as Iran, Turkey, Iraq, Pakistan, and sub-Saharan Africa are hyperendemic for anthrax, although the organism can be found in most areas of the world. Anthrax manifests disease in three different ways. Inhalation, gastrointestinal, and cutaneous anthrax
- 25 result from inhaling spores, ingesting spores in contaminated meat, or contacting spores in an open wound, respectively. Untreated inhalation or gastrointestinal anthrax has a case fatality rate of essentially 100 percent while cutaneous anthrax has a
- 30 case fatality rate of up to 25 percent. Previous research has shown that the protective antigen (PA) produced by *B. anthracis* can protect mice from anthrax. Even though the Anthrax vaccine is FDA licensed, reactogenicity is mild to moderate.

Therefore, there is a need for an efficacious vaccine for anthrax useful for protecting humans.

SUMMARY OF THE INVENTION

- 5 The present invention satisfies the need discussed above. The present invention relates to a method and composition for use in inducing an immune response which is protective against infection with anthrax.
- 10 In this vaccine strategy, a gene coding for a protein of interest is cloned into a VEE virus vector in place of the VEE virus structural genes; the result is a self-replicating RNA molecule, a replicon, that encodes its own replicase and transcriptase functions,
- 15 and in addition makes abundant quantities of mRNA encoding the foreign protein. When replicon RNA is transfected into eukaryotic cells along with two helper RNAs that express the VEE structural proteins (glycoproteins and nucleocapsid), the replicon RNA is
- 20 packaged into VEE virus-like particles by the VEE virus structural proteins, which are provided in trans. Since the helper RNAs lack packaging signals necessary for further propagation, the resulting VEE replicon particles (VRPs) which are produced are
- 25 infectious for one cycle but are defective thereafter. Upon infection of an individual cell with a VRP, an abortive infection occurs in which the infected cell produces the protein of interest in abundance, is ultimately killed by the infection, but does not
- 30 produce any viral progeny (Pushko et al., 1997, *Virology* 239, 389-401).
- The PA gene which contains a prokaryotic secretory signal and the entire 83 kDa coding sequence (Welkos et al., 1988, *Gene* 69, 287-300), was inserted

into the VEE replicon vaccine vector (Figure 1) and have demonstrated high level expression of this bacterial protein in eukaryotic cells in culture. Mice, either the C57BL/6 strain or the A/J strain,

5 inoculated with VRP containing the PA-replicon produced high specific antibody titers and were protected from developing anthrax when challenged subcutaneously with *B. anthracis*.

Therefore, it is one object of the present
10 invention to provide a VEE virus replicon vector comprising a VEE virus replicon and DNA fragments encoding the PA protein from *B. anthracis*.

It is another object of the present invention to provide a self replicating RNA comprising the VEE
15 virus replicon and any of the *B. anthracis* fragments described above.

It is another object of the present invention to provide infectious VEE virus replicon particles produced from the VEE virus replicon RNA described
20 above.

It is further an object of the invention to provide an immunological composition for the protection of mammals against *B. anthracis* infection comprising VEE virus replicon particles containing any
25 of the *B. anthracis* fragments described above or a combination of different VEE virus replicons each having a different *B. anthracis* fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

30 These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. Diagram of the protective antigen (PA)
35 replicon constructs. The PA replicons are similar to

the full-length VEE RNA except that the open reading frame encoding the VEE structural proteins was replaced with the PA genes.

Figure 2. Western blot of BHK cell lysates
5 showing expression of anthrax protective antigen from recombinant VEE replicons. a) uninfected cell lysate; b) recombinant expression product from *B. anthracis*; c) infected cell lysate.

10 Figure 3A and 3B. ELISA titers for mice (A, C57Bl/6 mice; B, A/J mice) immunized with PA-VRP. GMTs only includes those mice that survived challenge. a) Received 0.2 ml on day 0 and 28. b) Received 10⁷ iu on day 0 and 28. c) Received 10⁷ iu PA-VRP on day 0, 28, 15 56, and 84; C57Bl/6 mice that died, titer = 100; A/J mice that died, titer = 400. d) Received 10⁷ iu PA-VRP on day 0, 28, and 56; C57Bl/6 mice that died, titers = 400, 100, 100; A/J mice that died, titers = 102400, 100, 1600. e) Received 10⁷ iu PA-VRP on day 0, and 28; 20 C57Bl/6 mice that died, GMT = 1313; A/J mice that died, GMT = 1766. Mice were challenged 28 days after last inoculation with 18-32 LD₅₀ Sterne strain for C57Bl/6 and 12-25 LD₅₀ Sterne strain for A/J mice.

25 Figure 4. Schematic representation of constructs containing PA.

DETAILED DESCRIPTION

In the description that follows, a number of terms used in recombinant DNA, virology and immunology 30 are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Replicon. A replicon is equivalent to a full length virus from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site.

Transcription of the RNA from the replicon yields an RNA capable of initiating infection of the cell identically to that seen with the full-length

infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA *in trans*. This is typically done with two helpers also called defective helper RNAs. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for subgenomic mRNA transcription and the sequences for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the cell. The particles can then be inoculated into animals similar to parent virus. The replicon

particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only the viral nonstructural proteins and the product of the
5 heterologous gene cloned in place to the structural proteins.

The VEE virus replicon is a genetically reorganized version of the VEE virus genome in which the structural proteins genes are replaced with a gene
10 from an immunogen of interest, in this invention, the *B. anthracis* proteins. The result is a self replicating RNA (replicon) that can be packaged into infectious particles using defective helper RNAs that encode the glycoprotein and capsid proteins of the VEE
15 virus.

Subject. Includes both human, animal, e.g., horse, cattle, donkey, monkey, pig, dog, guinea pig, mouse, hamster, avian e.g., chicken, pheasant or turkey, fish and other marine animals, and insects
20 such as mosquito.

In one embodiment, the present invention relates to a recombinant DNA molecule that includes a VEE replicon and a DNA sequence encoding the non-toxic
25 protective antigen (PA) from *B. anthracis*. The sequence for PA has been determined and has been deposited in GenBank at accession no. M22589. The PA sequence encodes a prokaryotic secretory signal in addition to the entire sequence encoding the 83 Kd PA
30 (SEQ ID NO:1). Other nucleic acid sequences related to this invention include sequences wherein the secretory signal has been removed (MAT-PA) (SEQ ID NO:2), or replaced with other secretory signals known to people in the art such as the tissue plasminogen activator (TPA) secretory signal resulting in a DNA
35

fragment encoding TPA-PA (SEQ ID NO:3). Nucleic acid sequences included in this invention are sequences encoding the active form of PA, a 63 kDa protein, termed PA63 (SEQ ID NO:4). In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the *B. anthracis* proteins described and specified in SEQ ID NO:5 (TPA-PA), SEQ ID NO:6 (PA), SEQ ID NO:7 (MAT-PA), and SEQ ID NO:8 (PA63). Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*).

Nucleic acid molecules of the present invention may be in the form of RNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated

RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

- 5 The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein. Fragments include portions of the nucleotide sequences 10 of at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence is 15 position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence minus 1.
- 20 The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the *B. anthracis* polypeptides described above. Variants may occur naturally, such as a 25 natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occurring variants may be produced by known mutagenesis techniques. Such 30 variants include those produced by nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid 35 substitutions, deletions, or additions. Especially

preferred among these are silent substitutions, additions, and deletions which do not alter the properties and activities of the *B. anthracis* polypeptides disclosed herein or portions thereof.

- 5 Also preferred in this regard are conservative substitutions.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The
10 vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris*, or a virus vector such as for example, baculovirus vectors, retroviral vectors or adenoviral vectors, and others known in the art. The
15 cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences, or sequences which may be inducible and/or cell type-specific. Suitable promoters will be known to a person with
20 ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. When the DNA sequences described above are in a
25 replicon expression system, such as the VEE replicon described above, the proteins can be expressed *in vivo*. The DNA sequence for any of the *B. anthracis* proteins described above can be cloned into the multiple cloning site of a replicon such that
30 transcription of the RNA from the replicon yields an infectious RNA containing the *B. anthracis* protein or proteins of interest. Use of helper RNA containing sequences necessary for encapsulation of the viral transcript will result in the production of viral
35 particles containing replicon RNA which are able to

infect a host and initiate a single round of replication resulting in the expression of the *B. anthracis* proteins. Such replicon constructs include those listed in Table 1.

5 Table 1

Replicon

	Plasmid Name	expresses
10	p3014-TPA-PA	TPA signal, 5 amino acids of C terminal end of prokaryotic secretory signal, and the 83kDa mature protein.
15	p3014-PA	29 amino acid prokaryotic secretory signal, and the 83kDa mature protein secreted by <i>B. anthracis</i>
20	p3014-MAT-PA p3014-PA63	83kDa mature protein 63kDa protein (minus 20kDa N-terminal end that is naturally cleaved off by furin proteases at host cell membrane surface)
25	p3014-sub-domains	subdomain #1: from nucleotide 1891 to 2391; subdomain #2: from nucleotide 2392 to 2658; subdomain #3: from nucleotide 2659 to 3351; subdomain #4: from nucleotide 3352 to 3669; subdomain #5: from nucleotide 3670 to 4098 (Subdomains are numbered according to Welkos et al., 1988, supra, determined on subdomains present in the crystal structure, Petosa, et al., 1997, Nature 385, 833-838).
30		
35		

The VEE constructs containing anthrax proteins can be used as a DNA vaccine, or for the production of RNA molecules as described below.

40 In another embodiment, the present invention relates to RNA molecules resulting from the transcription of the constructs described above. The RNA molecules can be prepared by *in vitro* transcription using methods known in the art and described in the Examples below. Alternatively, the

RNA molecules can be produced by transcription of the constructs *in vivo*, and isolating the RNA. These and other methods for obtaining RNA transcripts of the constructs are known in the art. Please see Current

- 5 Protocols in Molecular Biology. Frederick M. Ausubel et al. (eds.), John Wiley and Sons, Inc. The RNA molecules can be used, for example, as a nucleic acid vaccine, or to transfect cells along with RNA from helper plasmids, one of which expresses VEE
- 10 glycoproteins and the other VEE capsid proteins, as described above, in order to obtain replicon particles.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection,

15 electroporation, infection, and other methods known in the art and described in standard laboratory manuals such as Current Protocols in Molecular Biology, Ausubel, F. M. et al. (Eds), Wiley & Sons, Inc. All documents cited herein supra and infra are hereby

20 incorporated in their entirety by reference thereto.

- In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences
- 25 when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions,
- 30

and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitzsch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of protein of the invention, such as glutathione S-transferase. The recombinant molecule can be suitable for transfecting eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as baby hamster kidney (BHK) cells, MRC-5 cells and vero cells, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase

expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of
5 DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein or polypeptide specified as amino acid sequence in SEQ ID NO:5 (TPA-PA), SEQ ID
10 NO:6 (PA), SEQ ID NO:7 (MAT-PA), and SEQ ID NO:8 (PA63).

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to
15 that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically
20 identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including
25 for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

30 The recombinant or fusion protein can be used as a vaccine for immunity against anthrax infection or as a diagnostic tool for detection of bacillus anthracis. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit
35 anthrax or *B. anthracis* proteins, such as host

proteins or chemically derived agents or other proteins which may interact with *B. anthracis* proteins of the present invention to inhibit its function. A method for testing the effectiveness of an anti-anthrax drug or agent can for example be the rat anthrax toxin assay (Ivins et al. 1984, *Infec. Immun.* 52, 454-458 and Ivins et al. 1986) or a skin test in rabbits for assaying antiserum against anthrax toxin (Belton and Henderson, 1956, *Br. J. Exp. Path.* 37, 156-160).

In another embodiment, the present invention relates to an anthrax vaccine comprising one or more replicon particles derived from one or more replicons encoding one or more *B. anthracis* proteins or polypeptides as described above. The present invention relates a method for providing immunity against anthrax said method comprising administering one or more replicon particles containing any combination of the *B. anthracis* proteins to a subject such that a protective immune reaction is generated.

Vaccine formulations of the present invention comprise an immunogenic amount of a replicon particle, resulting from one of the replicon constructs described above, or a combination of replicon particles as a multivalent vaccine, in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered. An amount of from about 10^2 to 10^7 per dose is suitable, more or less can be used depending upon the age and species of the subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the replicon particles disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular 5 injection), by in ovo injection in birds, orally and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the virus to an airway surface can be carried out by intranasal 10 administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable 15 particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the replicon as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering 20 respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is 25 administered.

When the replicon RNA or DNA is used as a vaccine, the replicon RNA or DNA can be administered directly using techniques such as delivery on gold beads (gene gun), delivery by liposomes, or direct 30 injection, among other methods known to people in the art. Any one or more constructs or replicating RNA described above can be used in any combination effective to elicit an immunogenic response in a subject. Generally, the nucleic acid vaccine 35 administered may be in an amount of about 1-5 ug of

nucleic acid per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine 5 to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in 10 which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and/or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent 15 dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses 20 expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

The following MATERIALS AND METHODS were used in the examples that follow.

Plasmids. Construction of the VEE replicon, 25 capsid (C-) helper, and glycoprotein (GP-) helper plasmids was previously described (Pushko, 1997, supra). The PA gene from nucleotide 1804 to 4098 (SEQ ID NO:1) (Welkos et al., 1988, supra) was cloned into the VEE replicon plasmid as a BamHI/BamHI fragment 30 utilizing a shuttle vector. TPA-PA (SEQ ID NO:3), MAT-PA (SEQ ID NO:2), and PA63 (SEQ ID NO:4) were PCR amplified using forward and reverse primers which also contained the ClaI recognition site. The PCR products were agarose gel purified and ligated into the T/A 35 vector pCR2.1 (Invitrogen, Inc.). The PA genes, after

digestion of pCR2.1-PA's with *Cla*I, were ligated into the VEE replicon plasmid. The Lassa nucleocapsid replicon (Lassa N-replicon) was constructed as previously described (Pushko, 1997, *supra*) and used as

5 a negative control replicon.

Production of VRP. Plasmid templates for the PA-replicons, C-helper, GP-helper, and the Lassa N-replicon were linearized by digestion with *Not*I at a unique site downstream from the coding sequences, and

10 capped run-off transcripts were prepared *in vitro* using T7 RNA polymerase. Packaging of the replicons into VEE replicon particles (VRPs) was accomplished by electroporating the replicon RNA and the two helper RNAs into BHK cells. VRPs were harvested between 20

15 and 27 hours after transfection and purified from cell culture supernatants by ultracentrifugation through a discontinuous sucrose gradient (20%). After reconstituting the pelleted VRP in 1/50 volume phosphate buffered saline, the VRPs were stored at -

20 70°C.

Analysis of expression products and titration of VRP.

Subconfluent monolayers were infected with PA-VRP's (either TPA-PA, PA, MAT-PA, or

25 PA63) or Lassa N-VRP (m.o.i. = 2) or cell suspensions were electroporated with replicon RNA. Cells were harvested at approximately 20-24 hours and expressed proteins were separated by SDS-PAGE. Visualization of PA proteins was accomplished using a chemiluminescence western blot assay and antibodies specific for each

30 protein. Titration of VRPs was accomplished by infecting subconfluent monolayers with increasing dilutions of purified VRP. Antigen positive cells were visualized in an indirect immunofluorescence assay using a monoclonal antibody specific for each protein,

or in a direct immunofluorescence assay using an FITC-conjugated monkey anti-Lassa serum.

Immunization of mice. Mice were inoculated 1 to 4 times at 28 day intervals with 10^5 to 10^7 infectious units (iu) of either PA-VRP or Lassa N-VRP (negative control). Positive control mice for the anthrax study were inoculated subcutaneously with 0.2 ml of anthrax vaccine at 28 day intervals. Serum for ELISA was obtained 2 days before each inoculation and 10 3 days before challenge.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with protective antigen (gift from Joe Farchaus, Bacteriology Division) (1 ug/ml) in PBS and allowed to absorb overnight at 4°C. 15 Four fold serum dilutions in blocking buffer were applied to the plates and incubated at 37°C for 1 hour. After washing, an anti-mouse secondary antibody (HRP conjugated) was added to the plate and incubated for an additional hour at 37°C. After washing, bound 20 antibody was detected colorimetrically using ABTS as a substrate.

Challenge of mice.

Anthrax challenge: C57BL/6 or A/J mice were challenged subcutaneously with 12-32 LD₅₀ *B. anthracis* (Sterne) 31 days after the last inoculation.

Example 1

Packaging and expression of PA-replicon
The PA-replicon was efficiently packaged into VRPs using the double helper system. Stock solutions 30 contained about 10^8 infectious units of purified PA-VRP per milliliter. No replication competent VEE virus was detected in any of the preparations. A western blot of cell lysates, generated from BHK cells infected with PA-VRP, demonstrates a product of

approximately 85 kDa (Figure 2). Because the PA-VRP expression product contains a 29 amino acid prokaryotic secretory signal, which is cleaved from the standard, the PA product from the BHK cells appears larger than the PA standard.

5 Example 2

Protection against challenge with *B. anthracis*

The results of the animal studies demonstrated that PA from *B. anthracis* could be used to immunize and protect mice from a lethal challenge of *B. anthracis* (Sterne strain). Although the Sterne strain is attenuated in some animals, it is virulent in mice. Controls in the experiment included mice vaccinated with the current human vaccine (anthrax vaccine absorbed, AVA) which protected all mice, and mice immunized with the Lassa N-replicon which expressed an antigen irrelevant to anthrax protection which failed to protect the mice. Figure 3A shows ELISA titers and survival for C57Bl/6 mice inoculated with two to four doses of PA-VRP. Seventy percent of the C57Bl/6 mice which received three inoculations of the PA-VRP were protected whereas 90% of the C57Bl/6 mice which received four inoculations were protected from an otherwise lethal challenge of *B. anthracis* (Sterne strain). The geometric mean of the serum ELISA titers to PA of C57Bl/6 mice given four doses of the PA-replicon was 150500, as compared to 264 for mice that were inoculated with the negative control replicon. Figure 3B shows ELISA titers and survival for A/J mice inoculated with two to four doses of PA-VRP. Three inoculations of the PA-VRP protected 70% of the A/J mice, whereas four inoculations protected 90% of the A/J mice from an otherwise lethal challenge of the *B. anthracis* (Sterne strain). The geometric mean of the serum ELISA titers against PA in A/J mice given four

doses of the PA-replicon was 223336 as compared to 100 for mice which were inoculated with the negative control replicon. As expected, none of the mice (from either strain) which were inoculated with the negative 5 control replicon survived challenge with *B. anthracis* (Sterne strain).

Discussion/Conclusion

Since VEE virus replicates in the cytoplasm of 10 eukaryotic cells, the VEE replicon vaccine vector is a useful tool for the expression of prokaryotic genes in eukaryotic cells. Cytoplasmic expression of genes alleviates the difficulties imposed by splicing and nuclear transport of mRNA. We used the VEE replicon as 15 a way to express the prokaryotic PA gene in eukaryotic cells and to develop a new vaccine candidate against anthrax.

The human AVA contains mostly PA protein and is presumed to protect humans by eliciting an antibody 20 response that can neutralize the PA portion of anthrax toxin produced by invading *B. anthracis*. After neutralization of the toxin, the immune system destroys the invading bacteria. In order to model the human disease, we chose to use mice and the Sterne 25 strain of *B. anthracis*. The Sterne strain produces anthrax toxin (encoded on the pXO1 plasmid) but lacks a capsule (encoded on the pXO2 plasmid). Mice exposed to large quantities of Sterne (ranging from 10^3 to 10^8 spores for the different mouse strains) are not able 30 to overcome the effects of the toxin produced by the bacteria so they succumb to infection. We hypothesized that by immunizing the mice with PA, thereby inducing neutralizing antibodies to the PA

portion of the toxin produced by a challenge inoculum, that the mice would be protected.

A VEE replicon was constructed which expressed the PA gene from *B. anthracis* and was evaluated for *in vitro* expression, *in vivo* immunogenicity, and protective efficacy. C57Bl/6 and A/J mice demonstrated increasing antibody responses to PA when inoculated with increasing doses of PA-VRP. The two mouse strains inoculated with up to four doses of PA-VRP displayed increased protection against a lethal challenge with *B. anthracis* (Sterne strain). The use of mice and the Sterne strain of *B. anthracis* useful as a model system for studying the immunogenicity and efficacy of replicon based anthrax vaccines.

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What is claimed is:

1. A recombinant DNA construct comprising:
 - (i) a vector, and
 - (ii) at least one nucleic acid5 fragment comprising any combination of *B. anthracis* proteins selected from the group consisting of PA, MAT-PA, TPA-PA, and PA63.
- 10 2. A recombinant DNA construct according to claim 1 wherein said vector is an expression vector.
- 15 3. A recombinant DNA construct according to claim 1 wherein said vector is a prokaryotic vector.
- 20 4. A recombinant DNA construct according to claim 1 wherein said vector is a eukaryotic vector.
5. The recombinant DNA construct of claim 1 wherein said vector is a VEE virus replicon vector.
- 25 6. The recombinant DNA construct according to claim 5 wherein said construct is p3014-PA.
7. The recombinant DNA construct according to claim 5 wherein said construct is p3014-TPA-PA.
- 30 8. The recombinant DNA construct according to claim 5 wherein said construct is p3014-MAT-PA.
9. The recombinant DNA construct according to claim 5 wherein said construct is p3014-PA63.
- 35 10. Self replicating RNA produced from a construct chosen from the group consisting of: p3014-PA, p3014-TPA-PA, p3014-MAT-PA, and p3014-P63

11. Infectious alphavirus particles produced from packaging the self replicating RNA of claim 10.
- 5 12. A pharmaceutical composition comprising infectious alphavirus particles according to claim 11 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.
- 10 13. A host cell transformed with a recombinant DNA construct according to claim 5.
- 15 14. A host cell according to claim 13 wherein said host cell is prokaryotic.
16. A method for producing *B. anthracis* protein comprising culturing the cells according to claim 14 under conditions such that said DNA fragment is expressed and said protein is produced.
- 20 17. A method for producing *B. anthracis* protein comprising culturing the cells according to claim 15 under conditions such that said DNA fragment is expressed and said protein is produced.
- 25 18. A vaccine for anthrax comprising viral particles containing one or more replicon RNA encoding any combination of *B. anthracis* proteins chosen from the group consisting of PA, PA-MAT, TPA-PA, and PA63.
- 30 19. A pharmaceutical composition comprising the self replication RNA of claim 14 in an effective

immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

20. A pharmaceutical composition comprising one
5 or more recombinant DNA constructs chosen from the group consisting of p3014-PA, p3014-TPA-PA, p3014-MAT-PA, and p3014-P63 in a pharmaceutically acceptable amount, in a pharmaceutically acceptable carrier and/or adjuvant.

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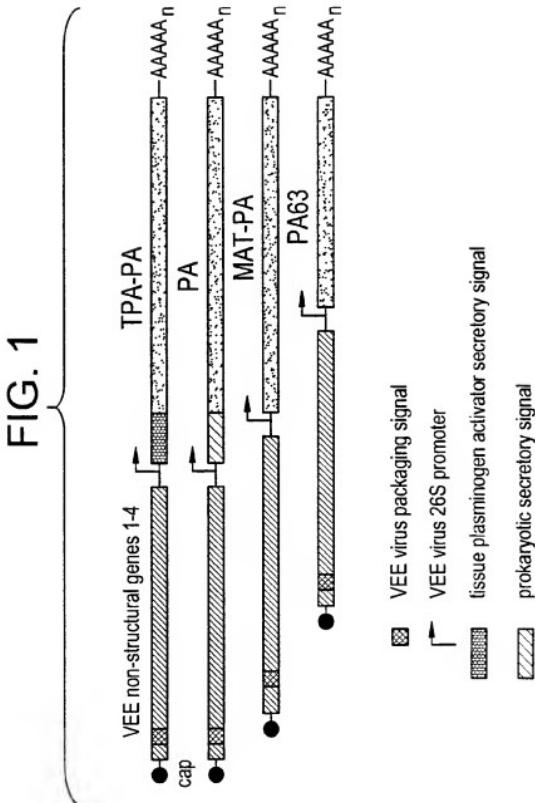


FIG. 2

FIG. 3A

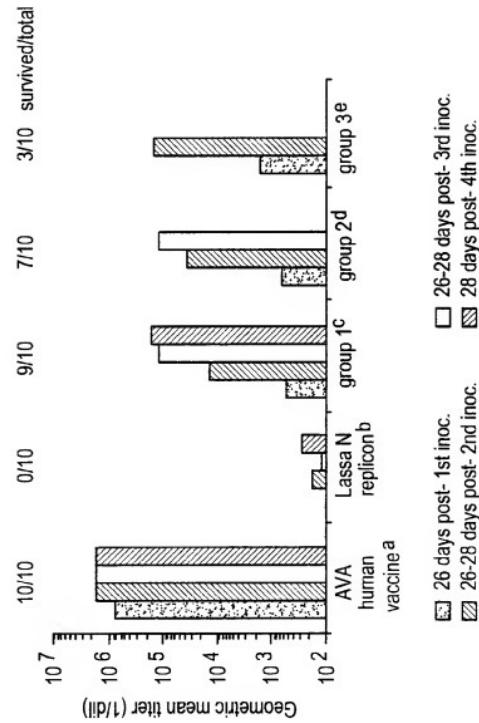
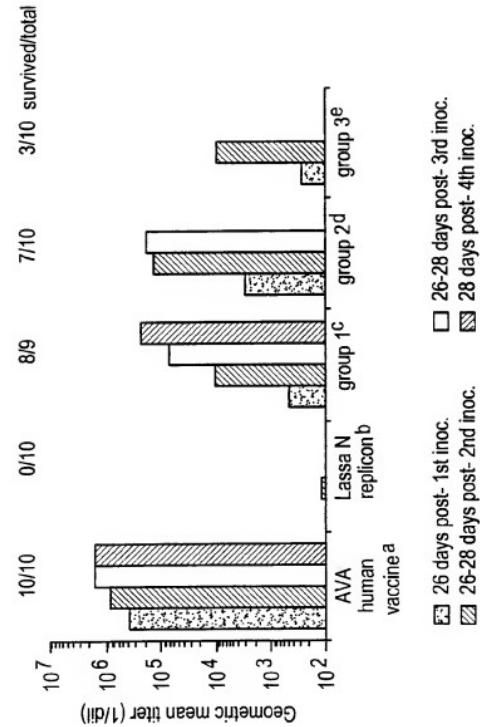
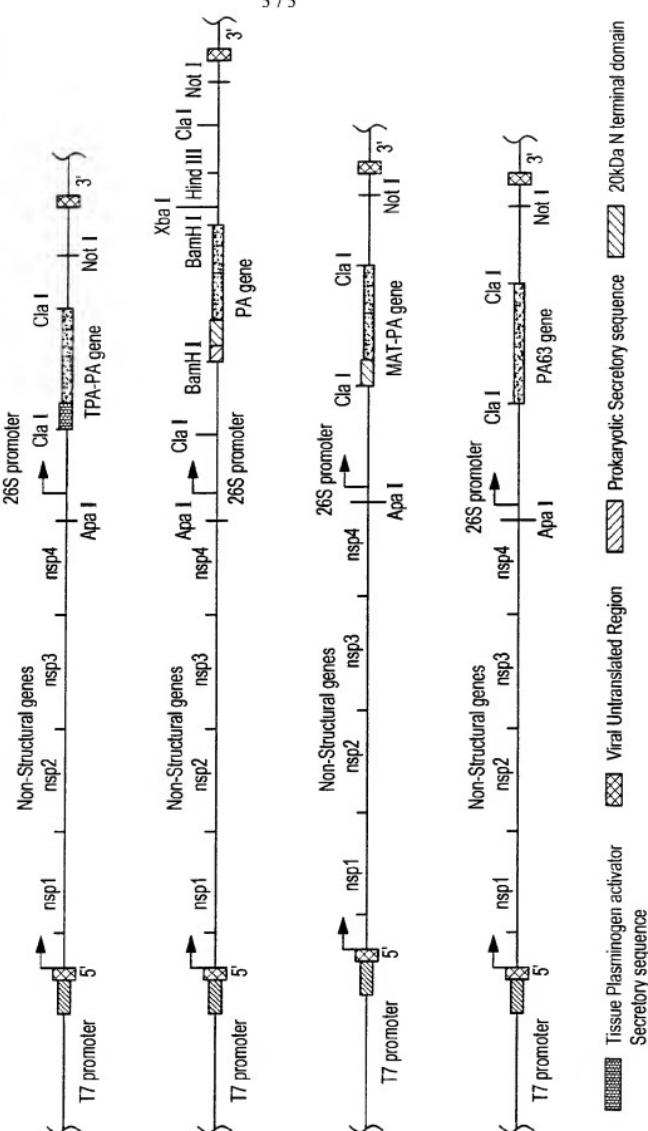


FIG. 3B



5 / 5

FIG. 4



SEQUENCE LISTING

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Infectious Diseases
Lee, John Scott
Pushko, Peter
Parker, Michael D.
Smith, Jonathan F.
Welkos, Susan L.

<120> Anthrax Vaccines

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 99/15568

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/32 A61K39/07 C12N7/01 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L.C. IACONO-CONNORS ET AL.,: "PROTECTION AGAINST ANTHRAX WITH RECOMBINANT VIRUS-EXPRESSED PROTECTIVE ANTIGEN IN EXPERIMENTAL ANIMALS", INFECTION AND IMMUNITY, vol. 59, no. 6, June 1991 (1991-06), pages 1961-1965, XP000916157 the whole document ---	1,2,4
Y	---	4-20 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"*&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

24 July 2000

02/08/2000

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Authorized officer

Julia, P

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/15568

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PUSHKO P ET AL: "Replicon-helper systems from attenuated Venezuelan Equine Encephalitis virus: Expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo" VIROLOGY, US, ACADEMIC PRESS, ORLANDO, vol. 239, no. 2, 22 December 1997 (1997-12-22), pages 389-401-401, XP002123951 ISSN: 0042-6822 the whole document ----	4-20
A	WO 96 17067 A (ZYMOGENETICS INC) 6 June 1996 (1996-06-06) page 14, line 18 -page 16, line 35 ----	1-5, 7, 10-20
X	SINGH Y ET AL: "A DELETED VARIANT OF BACILLUS ANTHRACIS PROTECTIVE ANTIGEN IS NON-TOXIC AND BLOCKS ANTHRAX TOXIN ACTION IN VIVO" JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 264, no. 32, 15 November 1989 (1989-11-15), pages 19103-19107, XP000650160 ISSN: 0021-9258 the whole document ----	1-3
Y	WO 95 07994 A (VIAGENE INC) 23 March 1995 (1995-03-23) page 9 lines 10-12, page 33 lines 31-35 and example 6 ----	4-20
X	IVINS B E ET AL: "CLONING AND EXPRESSION OF THE BACILLUS ANTHRACIS PROTECTIVE ANTIGEN GENE IN BACILLUS SUBTILIS" INFECTION AND IMMUNITY, US, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, vol. 54, no. 2, 1 November 1986 (1986-11-01), pages 537-542, XP000650052 ISSN: 0019-9567 the whole document ----	1-3
Y	WO 96 37616 A (UNIV NORTH CAROLINA ;US HEALTH (US); JOHNSTON ROBERT E (US); DAVIS) 28 November 1996 (1996-11-28) the whole document ----	4-20
		-/-

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/15568

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 08952 A (SECR DEFENCE ;BAILLIE LESLIE WILLIAM JAMES (GB)) 5 March 1998 (1998-03-05) the whole document -----	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/15568

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9617067	A 06-06-1996	US 5641655 A		24-06-1997
		AU 689373 B		26-03-1998
		AU 4737696 A		19-06-1996
		CA 2206399 A		06-06-1996
		CN 1171819 A		28-01-1998
		EP 0799317 A		08-10-1997
		NZ 301858 A		28-01-1999
WO 9507994	A 23-03-1995	AU 3682699 A		07-10-1999
		AU 703653 B		01-04-1999
		AU 5645098 A		04-06-1998
		AU 690583 B		30-04-1998
		AU 7835894 A		03-04-1995
		CA 2158937 A		23-03-1995
		EP 0694070 A		31-01-1996
		EP 0711829 A		15-05-1996
		EP 0716148 A		12-06-1996
		EP 0814154 A		29-12-1997
		EP 0982405 A		01-03-2000
		FI 954601 A		23-02-1996
		JP 9503657 T		15-04-1997
		NO 953901 A		04-01-1996
		US 6015686 A		18-01-2000
		US 5814482 A		29-09-1998
		US 5843723 A		01-12-1998
		US 5789245 A		04-08-1998
		US 6015694 A		18-01-2000
WO 9637616	A 28-11-1996	US 5792462 A		11-08-1998
		AU 5925696 A		11-12-1996
		CA 2220964 A		28-11-1996
		EP 1012295 A		28-06-2000
		JP 11505719 T		25-05-1999
WO 9808952	A 05-03-1998	EP 0927255 A		07-07-1999
		GB 2331523 A		26-05-1999